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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 Induces Heme Oxygenase-1 Gene Expression in a Reactive Oxygen Species-dependent Manner in Human Lymphocytes*

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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15dPGJ$_2$) has been proposed recently as a potent anti-inflammatory agent. However, the mechanisms by which 15dPGJ$_2$ mediates its therapeutic effects *in vivo* are unclear. We demonstrate that 15dPGJ$_2$ at micromolar (2.5–10 μM) concentrations induces the expression of heme oxygenase-1 (HO-1), an anti-inflammatory enzyme, at both mRNA and protein levels in human lymphocytes. In contrast, troglitazone and ciglitazone, two thiazolidinediones that mimic several effects of 15dPGJ$_2$ through their binding to the peroxisome proliferator-activated receptor (PPAR)-γ, did not affect HO-1 expression, and the positive effect of 15dPGJ$_2$ on this process was mimicked instead by other cyclopentenone prostaglandins (PG), such as PGD$_2$ (the precursor of 15dPGJ$_2$) and PGA$_1$ and PGA$_2$ which do not interact with PPAR-γ. Also, 15dPGJ$_2$ enhanced the intracellular production of reactive oxygen species (ROS) and increased xanthine oxidase activity *in vitro*. Inhibition of intracellular ROS production by *N*-acetylcysteine, TEMPO, Me$_2$SO, 1,10-phenanthroline, or allopurinol resulted in a decreased 15dPGJ$_2$-dependent HO-1 expression in the cells. Furthermore, buthionine sulfoximine, an inhibitor of reduced glutathione synthesis, or Fe^{2+}/Cu^{2+} ions enhanced the positive effect of 15dPGJ$_2$ on HO-1 expression. On the other hand, the inhibition of phosphatidylinositol 3-kinase or p38 mitogen-activated protein kinase, or the blockade of transcription factor NF-κB activation, hindered 15dPGJ$_2$-elicited HO-1 expression. Collectively, the present data suggest that 15dPGJ$_2$ anti-inflammatory actions at pharmacological concentrations involve the induction of *HO-1* gene expression through mechanisms independent of PPAR-γ activation and dependent on ROS produced via the xanthine/xanthine oxidase system and/or through Fenton reactions. Both phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase signaling pathways also appear implicated in modulation of HO-1 expression by 15dPGJ$_2$.

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (herein referred to as 15dPGJ$_2$)[1] is an active metabolite derived from PGD$_2$ dehydration *in vivo*, which is physiologically present in body fluids at 10^{-12} to 10^{-9} M concentrations. However, 15dPGJ$_2$ amounts become highly increased in several pathological conditions, such as infection and inflammation (1, 2). PGD$_2$ and thus 15dPGJ$_2$ are abundantly produced by mast cells, platelets, and alveolar macrophages, and 15dPGJ$_2$ has been proposed as a key immunoregulatory lipid mediator (3–5). A number of its actions seem to be exerted through its binding to peroxisome proliferator-activated receptor (PPAR)-γ (6–8). The PPARs are nuclear hormone receptors that can bind, normally as heterodimers with the retinoid X receptor, to peroxisome proliferator-responsive elements (AGGTCA$_n$AGGTCA) present in the promoters of their target genes to activate their transcription (9). A variety of immune cells, including T and B lymphocytes as well as macrophages, have been found to express PPAR-γ and their activation to be regulated via PPAR-γ-dependent mechanisms (7, 8, 10). However, it has been also demonstrated that other effects of 15dPGJ$_2$ are exerted independently of PPAR-γ (reviewed in Ref. 1), such as the activation of mitogen-activated protein (MAP) kinase cascades in astrocytes and preadipocytes, which is mediated by reactive oxygen species (ROS) (11), the induction of caspase-dependent apoptosis in eosinophils and neutrophils (12), the inhibition of cell adhesion and the oxidative burst in neutrophils (13), and the induction of the expression of proinflammatory genes in monocytes/macrophages derived from PPARγ-deficient embryonic stem cells (14, 15).

The role of 15dPGJ$_2$ in the modulation of inflammation is nevertheless complex, because this prostaglandin exhibits both anti-inflammatory and proinflammatory functions and thus remains the subject of intensive investigation. In this context, 15dPGJ$_2$ has been shown to inhibit the expression of inducible NO synthase, as well as tumor necrosis factor-α and interleukin-1β production in mouse and human macrophages, thus suggesting a role for 15dPGJ$_2$ in the inhibition of inflammation (16, 17). Available data also suggest that the induction of heme

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[1] The abbreviations used are: 15dPGJ$_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; PPAR, peroxisome proliferator-activated receptor; MAP, mitogen-activated protein; ROS, reactive oxygen species; HO-1, heme oxygenase-1; NAC, *N*-acetylcysteine; BSO, buthionine sulfoximine; HMAP, 4-hydroxy-3-methoxyacetophenone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DCFDA, 2,7-dichlorohydrofluorescein diacetate; O_2^-, superoxide anion; $^{\cdot}$OH, hydroxyl radicals; ERK1/2, extracellular signal-regulated kinases 1 and 2; PI, phosphatidylinositol; RT, reverse transcriptase.

oxygenase-1 (HO-1) expression elicited by Δ^{12}-PGJ$_2$ in rat basophilic leukemia cells and other cell types involved in the inflammatory response may play an important role in the fate of heme liberated during inflammation, and thus an anti-inflammatory role has been ascribed to Δ^{12}-PGJ$_2$ (18). In this view, 15dPGJ$_2$ has been suggested to play a feedback regulatory role in the resolution phase of inflammation and hence is considered as a potential therapeutic target for the treatment of inflammatory diseases (1). However, caution must be taken in assigning a solely anti-inflammatory role to 15dPGJ$_2$, because there is also experimental evidence that 15dPGJ$_2$ can induce the synthesis of the type II-secreted proinflammatory mediators phospholipase A (6) and COX-2 in smooth muscle (20) and epithelial cells (21). Moreover, it has been shown recently (22) that in stimulated human T lymphocytes, 15dPGJ$_2$ induces a significant increase in interleukin-8 production through the activation of a MAP kinase- and NF-κB-dependent signaling pathway.

Heme oxygenase is a widely distributed enzyme in mammalian tissues whose main function is associated with the degradation of heme to biliverdin, iron, and carbon monoxide (23). Three distinct isoforms of this protein have been characterized, two of which (HO-2 and HO-3) are constitutively expressed, whereas the third (HO-1) displays inducible expression (24). Several pathological states, including hypoxia, atherosclerosis, and inflammation, have been found to be accompanied by overexpression of the *HO-1* gene and increased HO-1 enzyme activity (25–27). Over the past few years, several studies (28, 29) have revealed the important function of HO-1 as a cytoprotective mechanism against oxidative insults, derived from the antioxidant activities of biliverdin and bilirubin and from the anti-inflammatory action of carbon monoxide. In this context, a common denominator characterizing the prompt stimulation of HO-1 expression occurring under the above conditions is a drastic change in the intracellular redox status accompanied by a transient decrease in cytosolic reduced glutathione levels (25, 30).

Recent studies have illustrated that 15dPGJ$_2$ induces HO-1 expression in a variety of cells, including hepatocytes (31), cardiac myocytes (32), microglial cells (33), murine macrophages (34), and rat basophilic leukemia cells (18). Also, it has been shown that 15dPGJ$_2$ is able to induce apoptosis in mouse T cells and to inhibit T cell activation (7, 8, 35). However, the effect of 15dPGJ$_2$ on *HO-1* gene expression has not yet been analyzed in human lymphocytes, and in general there is no information available on the signaling mechanisms modulating HO-1 synthesis in these cells. In the present study, we have investigated the effect of 15dPGJ$_2$, using pharmacological concentrations, on the expression of HO-1 in human lymphocytes, and the involvement of ROS-dependent specific pathways in this process. We present clear evidence that 15dPGJ$_2$ at micromolar (2.5–10 μM) levels induces an increase of HO-1 mRNA and protein levels in human lymphocytes, a process that is prevented by scavengers of ROS, and we postulate that ROS derived from both Fenton chemistry and the xanthine/xanthine oxidase system are implicated in the induction of *HO-1* gene expression in this cell type. In addition, we provide evidence of phosphatidylinositol 3-kinase (PI3-kinase) and p38 MAP kinase acting as mediators of 15dPGJ$_2$ effects on HO-1 expression and of the involvement of the transcription factor NF-κB in this process. These results contribute to shed some light on the mechanisms whereby 15dPGJ$_2$ modulates leukocyte functions through PPAR-γ-independent pathways.

EXPERIMENTAL PROCEDURES

Materials—15dPGJ$_2$, SN50 (AAVALLPAVLLALLAPVQRKRQKLMP), and SN50M (AAVALLPAVLLALLAPVQRNGQKLMP) peptides were obtained from Biomol (Plymouth Meeting, PA). Troglitazone, ciglitazone, Wy14,643, PGD$_2$, PGA$_1$, and PGA$_2$ were products of Cayman Chemical (Ann Arbor, MI). Sodium arsenite, actinomycin D, cycloheximide, *N*-acetylcysteine (NAC), buthionine sulfoximine (BSO), TEMPO, Me$_2$SO, 1,10-phenanthroline, allopurinol, 4-hydroxy-3-methoxyacetophenone (HMAP), probenecid, luminol, lucigenin, ketoconazole, rotenone, genistein, staurosporine, cyclosporin A, okadaic acid, wortmannin, and LY294002 were purchased from Sigma. SB203580 and PD098059 were products of Calbiochem, and RPMI 1640 medium was obtained from Biomedia (Boussens, France).

Isolation and Culture of Human Lymphocytes—Human peripheral blood lymphocytes were isolated from fresh heparinized blood of healthy human donors, after informed consent, by Ficoll-Paque (Amersham Biosciences) gradient centrifugation followed by hypotonic lysis of residual erythrocytes (36). After elimination of adherent cells by incubation on plastic dishes for 45 min at 37 °C, lymphocytes were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained at 37 °C in an atmosphere of 5% CO_2. In all control experiments in which 15dPGJ$_2$ was omitted, its vehicle (0.1% Me$_2$SO) was added instead.

Western Blotting Analysis of HO-1 Protein Levels—Cells were rinsed once with ice-cold phosphate-buffered saline, resuspended in a lysis solution containing 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 50 mM NaF, 10% glycerol, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride, and maintained on ice for 30 min. Then the cells were disrupted by sonication on ice and, after centrifugation at 12,000 × *g* for 5 min at 4 °C, the protein concentration in the supernatant was determined by the Bradford method (37), using bovine serum albumin as a standard. Cell lysate proteins (38) were boiled in Laemmli loading buffer, resolved by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Pall, Madrid, Spain). Blots were probed with rabbit polyclonal antibodies to HO-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:1000 dilution, and horseradish peroxidase-conjugated secondary antibodies to rabbit IgG (Sigma) were used at a 1:5000 dilution to detect the 32-kDa HO-1 band by subsequent enhanced chemiluminescence (39). To verify even protein loading, the blots were subsequently stripped and reprobed with rabbit polyclonal antibodies against β-actin (42 kDa) from Santa Cruz Biotechnology (1:1000 dilution). Relative protein levels were determined by scanning densitometry analysis using the Scion Image software. Values given below each panel in Figs. 1–4 and 6–9 represent the mean (in relative units), normalized to control values, of at least three experiments performed with similar results.

RT-PCR Analysis of HO-1 mRNA Levels—Total cellular RNA was extracted using the phenol-guanidinium isothiocyanate method (40). Reverse transcription to cDNA was performed at 37 °C for 1 h in 24 μl of reaction mixture containing 2 μg of RNA, the four dNTPs at 1 mM each, 2.5 μM random hexamer primers, 1 mM dithiothreitol, 20 units of RNAsin ribonuclease inhibitor, and 100 units of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). PCR amplification of cDNA (4) was performed in 10 μl of reaction using the following HO-1-specific primers at 0.5 μM each: forward, 5′-TTCTTCACCTTCCCCAAC-3′, and reverse, 5′-GCATAAAGCCCTACAGCAAC-3′), the four dNTPs at 400 μM each, 5% Me$_2$SO, and 1 unit of *Taq*DNA polymerase (Roche Diagnostics). After an initial denaturation step at 94.5 °C for 5 min, PCR was performed for a total of 45 cycles, each at 94.5 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min. To ensure that equal amounts of cDNA were added to the PCRs, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified as a control, using the following primers: forward, 5′-CCACCCATGGCAAATTCCATGGC-3′, and reverse, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. PCR-amplified bands (HO-1, 365 bp; GADPH, 600 bp) were visualized on 1% agarose gels stained with ethidium bromide using the BioDoc-It™ system (Ultraviolet Products, Upland, CA).

ROS Production Assay—2,7-Dichlorohydrofluorescein diacetate (DCFDA, from Sigma) was used as an indicator of the amount of intracellular ROS (41). Briefly, 5 × 10^6 human lymphocytes were suspended in 1 ml of KR-Hepes and incubated in the presence of 2.5 μM DCFDA and 2.5 mM probenecid (an inhibitor of anion membrane transport) at 37 °C for 1 h in the dark. The cells were then rinsed twice with KR-Hepes plus 2.5 mM probenecid, and after addition of the stimulus, fluorescence intensity was measured for up to 4 h in a Wallac 1420 VICTOR^2$_{TM}$ spectrofluorometer, using excitation and emission wavelengths of 485 and 535 nm, respectively. In addition, luminol- and lucigenin-enhanced chemiluminescence assays were performed as described (42) for the measurement of total ROS and superoxide anion ($O_2^{\cdot-}$) levels, respectively.

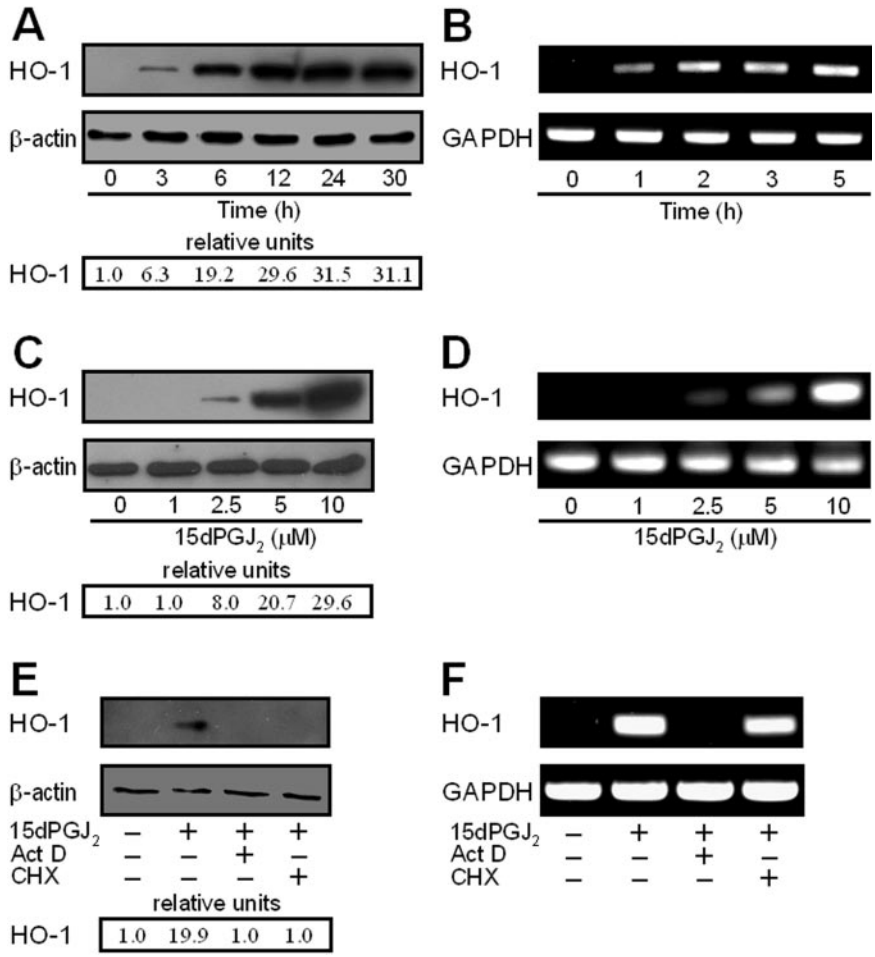

FIG. 1. **Induction by 15dPGJ$_2$ of HO-1 expression in human lymphocytes.** Cells were incubated at 37 °C with 10 μM 15dPGJ$_2$ for the indicated times (*A* and *B*) or at the indicated doses for 18 (*C*) or 4 h (*D*), or else were preincubated in the absence or presence of 5 μg/ml actinomycin D (*Act D*) or 5 μg/ml cycloheximide (*CHX*) for 1 h, and then treated or not with 5 μM 15dPGJ$_2$ for 18 (*E*) or 4 h (*F*). Thereafter, the cells were lysed, and HO-1 and β-actin protein levels were determined by Western blotting analysis (*A*, *C*, and *E*), and HO-1 and GAPDH mRNA levels were analyzed by RT-PCR (*B*, *D*, and *F*).

Xanthine Oxidase Activity Assay—Xanthine oxidase activity was determined by monitoring the rate of uric acid formation (43). Assays were performed using different concentrations of cow milk xanthine oxidase (Roche Diagnostics) in a reaction mixture (1 ml) containing 0.1 mM xanthine and 0.1 mM EDTA in 50 mM phosphate potassium buffer (pH 7.4). The reactions were carried out at 37 °C and stopped at 20 min by the addition of 100 μl of 100% (w/v) trichloroacetic acid. After centrifugation at 10,000 × *g* for 15 min, the uric acid produced was measured in the supernatants on the basis of the change in the absorbance at 293 nm.

RESULTS

15dPGJ$_2$ Induces HO-1 Gene Expression in Human Lymphocytes—Preliminary experiments were addressed to analyze the effect of 15dPGJ$_2$ on the expression of HO-1 in human lymphocytes. Fig. 1*A* shows that 10 μM 15dPGJ$_2$ elicited the synthesis of HO-1 protein in a time-dependent manner. This effect was noticeable at 3 h after 15dPGJ$_2$ addition, reaching its maximal expression at 12 h of treatment and maintaining at this level during the rest of the experiment (up to 30 h). The induction of HO-1 protein expression correlated with increased levels of HO-1 mRNA, which became detectable as early as 1 h after treatment with 15dPGJ$_2$ (Fig. 1*B*). A dose-dependent analysis showed that *HO-1* gene expression was induced at both protein (Fig. 1*C*) and mRNA (Fig. 1*D*) levels by concentrations of 15dPGJ$_2$ in the 2.5–10 μM range. In order to study the molecular mechanisms implicated in 15dPGJ$_2$-dependent HO-1 induction, human lymphocytes were treated with actinomycin D, a well known inhibitor of transcription, and cycloheximide, an established inhibitor of protein synthesis, before the addition of 15dPGJ$_2$ (Fig. 1, *E* and *F*). As expected, the pretreatment of lymphocytes with actinomycin D blocked both HO-1 protein and mRNA synthesis, whereas cycloheximide inhibited HO-1 protein expression but did not affect mRNA expression, suggesting that 15dPGJ$_2$ induction of HO-1 expression is exerted at the transcriptional level. As reported, 15dPGJ$_2$ can act on cells through either PPAR-γ-dependent or -independent mechanisms (13, 15). To address which was the case for HO-1 expression in human lymphocytes, we treated these cells with the PPAR-γ natural ligand, 15dPGJ$_2$, or with the synthetic PPAR-γ agonists, troglitazone and ciglitazone, and well as with the PPAR non-related inducer of HO-1 expression, sodium arsenite (44). Although both 15dPGJ$_2$ and arsenite and clearly promoted *HO-1* gene expression at both protein and mRNA levels (Fig. 2, *A* and *B*, respectively), neither troglitazone nor ciglitazone affected these processes. We also observed that Wy14,643, a PPAR-α agonist, was unable to promote HO-1 protein or mRNA expression in these cells (Fig. 2, *A* and *B*). These data strongly suggested that the induction of HO-1 expression by 15dPGJ$_2$ in human lymphocytes is exerted by a mechanism independent of PPAR activation.

Because the effects of cyclopentenone prostaglandins, like 15dPGJ$_2$ and A series prostaglandins, are mediated by the reactive α,β-unsaturated carbonyl group in their cyclopentane ring (45), subsequent experiments were addressed to study the effect of prostaglandins A, which do not bind to any member of the PPAR subfamily of receptors, on HO-1 expression. Fig. 2, *C* and *D,* illustrates that both HO-1 protein and mRNA levels were increased by both PGA$_1$ and PGD$_2$, the precursor of 15dPGJ$_2$ (46), in human lymphocytes. However, the effect of PGA$_2$ on HO-1 expression only was only detected on mRNA levels but not on protein levels.

15dPGJ$_2$ Induces HO-1 Expression through Changes in Intracellular ROS Levels—HO-1 expression has been shown to

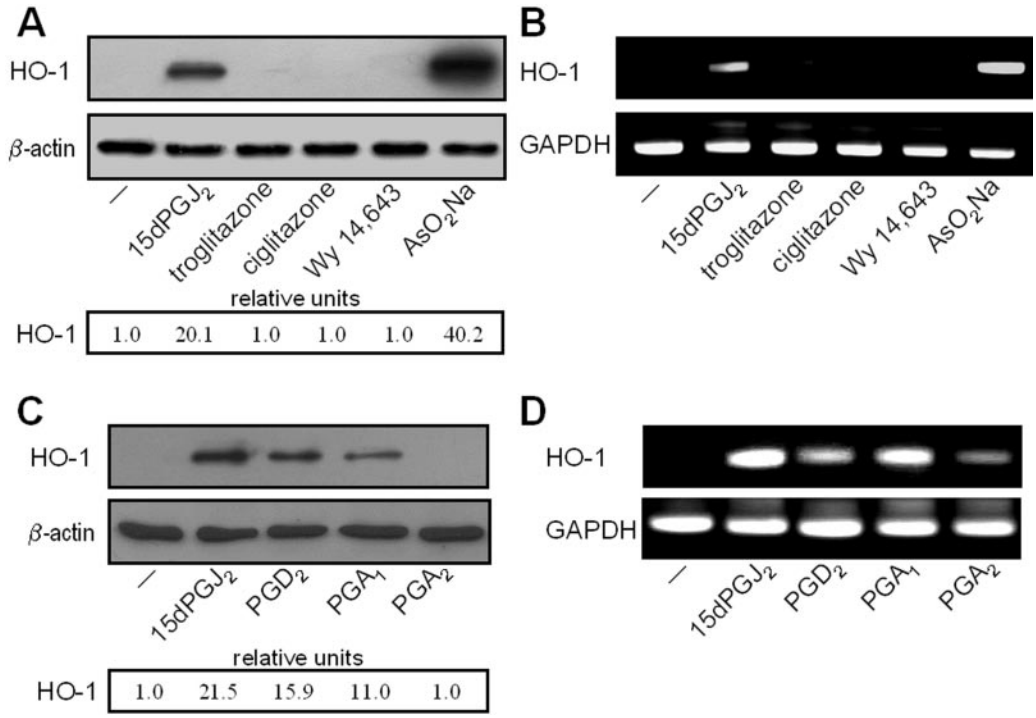

FIG. 2. **Effect of synthetic PPAR agonists and cyclopentenone prostaglandins on HO-1 expression in human lymphocytes.** Cells were incubated at 37 °C in the absence or presence of 5 μM 15dPGJ$_2$, 25 μM troglitazone, 40 μM ciglitazone, 25 μM Wy14,643, 5 μM sodium arsenite (*AsO$_2$Na*), 10 μM PGD$_2$, 15 μM PGA$_1$ or 25 μM PGA$_2$ for 18 (*A* and *C*) or 4 h (*B* and *D*). The cells were then lysed, and HO-1 and β-actin protein levels were determined by Western blotting analysis (*A* and *C*), and HO-1 and GAPDH mRNA levels were analyzed by RT-PCR (*B* and *D*).

become up-regulated in response to cellular stress or changes in the intracellular redox status in endothelial cells and fibroblasts (25, 30, 47). We thus tested whether the positive effect of 15dPGJ$_2$ on HO-1 expression was modified by NAC, a well known scavenger of ROS. With this goal, cultured lymphocytes were pretreated with NAC prior to the addition of 15dPGJ$_2$ to the medium. Fig. 3*A* illustrates that 0.5 mM NAC inhibited 15dPGJ$_2$-dependent HO-1 expression by about 60% and that this inhibition was complete at a dose of 5 mM NAC. Because it has also been described that a transient decrease in cellular reduced glutathione levels promotes HO-1 synthesis in a variety of mammalian (including human) cell types (48, 49), this observation prompted us to examine the effect of the pretreatment with BSO, an inhibitor of glutathione synthesis, on 15dPGJ$_2$-induced HO-1 expression. As shown in Fig. 3*B*, cells preincubated with BSO experienced a significantly higher 15dPGJ$_2$-induced HO-1 expression than did previously untreated cells. BSO, however, was by itself unable to induce HO-1 expression. To assess further whether 15dPGJ$_2$-dependent HO-1 expression was mediated by intracellular ROS, human lymphocytes were treated with two well known ROS scavengers, such as TEMPO, a superoxide dismutase mimetic for $O_2^{\cdot-}$, and Me$_2$SO at a concentration (0.5%) where it acts as a scavenger of hydroxyl radicals ($^{\cdot}$OH) (50, 51). As shown in Fig. 4, both of these reagents were found to drastically cancel 15dPGJ$_2$-elicited HO-1 expression. Subsequent experiments were addressed to analyze whether 15dPGJ$_2$ was able to alter intracellular ROS levels in lymphocytes. With this goal, the cells were treated with 15dPGJ$_2$, and changes in the intracellular levels of ROS were detected using the ROS-sensitive fluorescent probe DCFDA, an established sensor of oxidative stress (41). Fig. 5 shows that in human lymphocytes incubated with 15dPGJ$_2$, a higher production of ROS took place than in control cells treated with vehicle alone (0.1% Me$_2$SO), in which an unspecific increase of ROS levels was observed. It must be mentioned that Me$_2$SO, when used at a 0.1% concentration, neither affected cell viability nor did it act as an ROS scavenger (data not shown). In additional experiments performed using luminol- or lucigenin-enhanced chemiluminescence to measure total ROS and $O_2^{\cdot-}$ levels, respectively, similar results were obtained regarding 15dPGJ$_2$ effects (data not shown). This set of data indicates that intracellular ROS are involved in the induction of HO-1 expression by 15dPGJ$_2$ in human lymphocytes.

Effect of Fe^{2+}/Cu^{2+} Ions on 15dPGJ$_2$-induced HO-1 Expression—As in other cell types, in lymphocytes the most likely mode of intracellular production of highly active oxygen species, such as $^{\cdot}$OH, is via Fenton chemistry, which involves the reduction of H_2O_2 by Fe^{2+} or Cu^{+2} ions according to the reaction: $H_2O_2 + Fe^{2+} \leftrightarrows {}^{\cdot}OH + OH^- + Fe^{3+}$ (52). We thus analyzed whether chemical species generated from H_2O_2 through Fenton reactions could affect 15dPGJ$_2$-dependent HO-1 expression. Fig. 6 illustrates that the addition of Fe^{2+}/Cu^{2+} ions to lymphocytes prior to the treatment with 15dPGJ$_2$ notably enhanced HO-1 expression. Noteworthy, when the cells were incubated with Fe^{2+}/Cu^{2+} alone, a detectable expression of HO-1 protein also took place, although at lower levels than in the simultaneous presence of 15dPGJ$_2$. By contrast, the pretreatment of cells with 1,10-phenanthroline, a chelator of copper and iron ions, resulted in the cancellation of 15dPGJ$_2$-elicited HO-1 synthesis. These data indicate that ROS generated via Fenton reactions could constitute an efficient mechanism mediating the positive effect of 15dPGJ$_2$ on HO-1 induction.

Effect of the Xanthine/Xanthine Oxidase System on 15dPGJ$_2$-induced HO-1 Expression—The NADPH oxidase complex, the xanthine/xanthine oxidase system, and the mitochondrial respiratory chain are largely accredited as generator systems of intracellular ROS in most cell types (53, 54). When the xanthine oxidase inhibitor, allopurinol (55), was added to lymphocyte cultures prior to 15dPGJ$_2$ treatment, the expression of HO-1 became significantly decreased in a dose-dependent man-

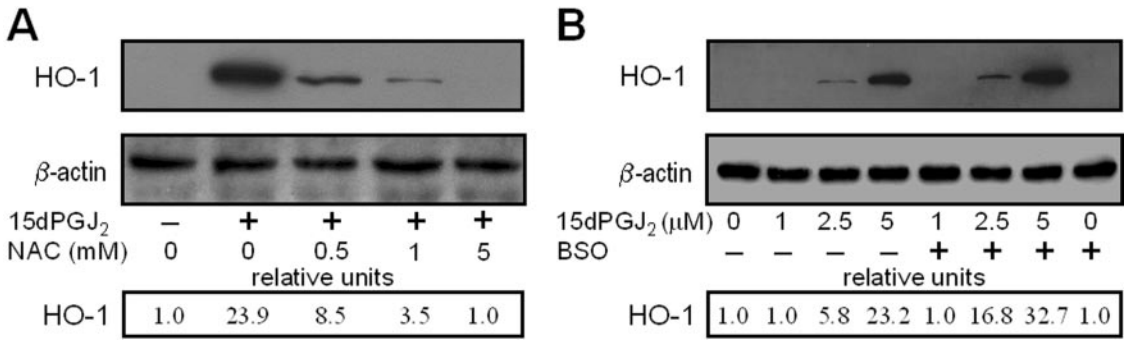

FIG. 3. **Effect of NAC and BSO on 15dPGJ$_2$-dependent HO-1 expression in human lymphocytes.** Cells were preincubated at 37 °C for 1 h with the indicated doses of NAC for 1 h and then treated or not with 5 μM 15dPGJ$_2$ for 18 h (*A*) or preincubated with 25 μM BSO for 1 h, and then treated with the indicated doses of 15dPGJ$_2$ for 18 h (*B*). Thereafter, the cells were lysed, and HO-1 and β-actin protein levels were determined by Western blotting analysis.

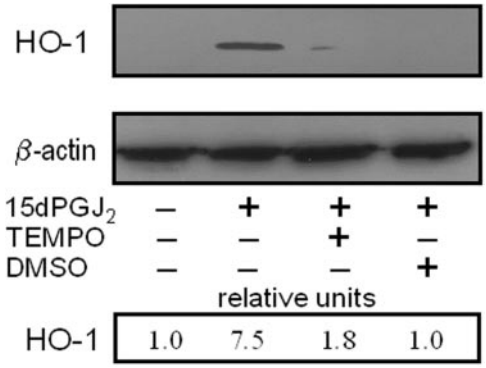

FIG. 4. **Effect of ROS scavengers on 15dPGJ$_2$-dependent HO-1 expression in human lymphocytes.** Cells were preincubated at 37 °C in the absence or presence of 100 μM TEMPO or 0.5% Me_2SO for 2 h and then treated or not with 3 μM 15dPGJ$_2$ for 18 h. Thereafter, the cells were lysed, and HO-1 and β-actin protein levels were determined by Western blotting analysis.

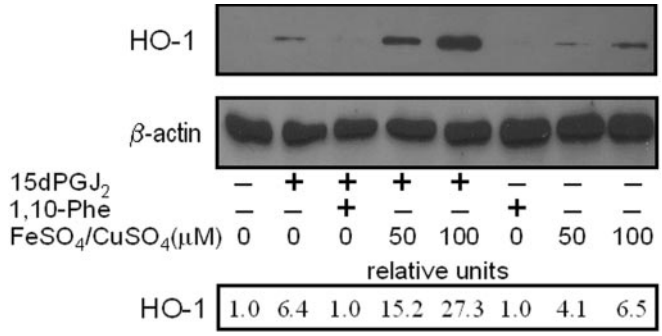

FIG. 6. **Effect of Fe^{2+}/Cu^{2+} ions on 15dPGJ$_2$-dependent HO-1 expression in human lymphocytes.** Cells were preincubated at 37 °C in the absence or presence of 20 μM 1,10-phenanthroline (*1,10-Phe*) or $FeSO_4$ plus $CuSO_4$ at the indicated doses for 1 h, and then treated or not with 3 μM 15dPGJ$_2$ for 18 h. Thereafter, the cells were lysed, and HO-1 and β-actin protein levels were determined by Western blotting analysis.

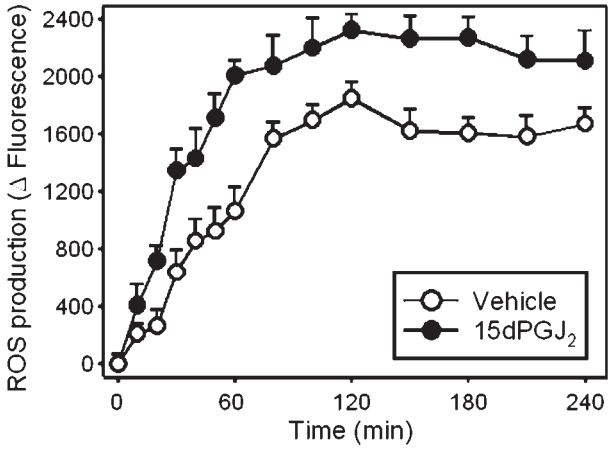

FIG. 5. **Effect of 15dPGJ$_2$ on intracellular ROS levels in human lymphocytes.** Cells were preincubated at 37 °C with 2.5 μM DCFDA for 1 h. They were then washed twice, and vehicle (0.1% Me_2SO) or 15dPGJ$_2$ (9) was added, and the fluorescence at 535 nm was recorded for different times up to 4 h. Plotted values are the mean ± S.E. from three separate experiments.

ner (Fig. 7*A*). However, other inhibitors of enzyme systems involved in ROS production, like rotenone (an inhibitor of mitochondrial electron transport), ketoconazole (a cytochrome P-450 inhibitor) and HMAP (an NADPH oxidase inhibitor), did not affect 15dPGJ$_2$-dependent HO-1 expression (data not shown). The inhibitory effect of allopurinol suggested the possibility that 15dPGJ$_2$ induction of HO-1 expression could be mediated by the activation of the xanthine/xanthine oxidase system. To test this hypothesis, we tried to assay xanthine oxidase activity in lymphocytes in the absence and presence of 15dPGJ$_2$. However, this enzyme activity was too low in this cell type, as reported previously (56), to allow reliable measurements by using our methodology. A different *in vitro* experimental approach was thus used, in which the effect of 15dPGJ$_2$ on a commercial preparation of xanthine oxidase was tested. It was found that upon incubation with 15dPGJ$_2$, the activity of this enzyme became notably increased, by about 10-fold (Fig. 7*B*). Present data thus suggest that a potential source for the ROS involved in 15dPGJ$_2$-dependent HO-1 expression in human lymphocytes could be xanthine oxidase.

15dPGJ$_2$ Induction of HO-1 Expression Is Mediated by PI3-kinase and MAP Kinase but Not by Protein Phosphatases 2A and 2B—The participation of these signaling molecules on the 15dPGJ$_2$ positive effect on HO-1 synthesis was analyzed by using specific pharmacological inhibitors. A relationship between ROS intracellular levels and the activity of both PI3-kinase and MAP kinases is well established (42, 57), and thus subsequent experiments were addressed to investigate the potential contribution of their mediated signaling pathways on 15dPGJ$_2$-dependent HO-1 expression. Toward this end, human lymphocytes were pretreated with wortmannin or LY294002, two inhibitors of PI3-kinase, and then stimulated with 15dPGJ$_2$ for 4 h (corresponding to the half-life of wortmannin in the culture medium). Fig. 8*A* illustrates that both PI3-kinase inhibitors significantly blocked the 15dPGJ$_2$ induction of HO-1 protein expression. Likewise, the potential involvement of MAP kinase pathways in the induction of HO-1 expression by 15dPGJ$_2$ was addressed by using SB203580, a p38 MAP kinase inhibitor, and PD098059, an inhibitor of MEK1/2, the up-

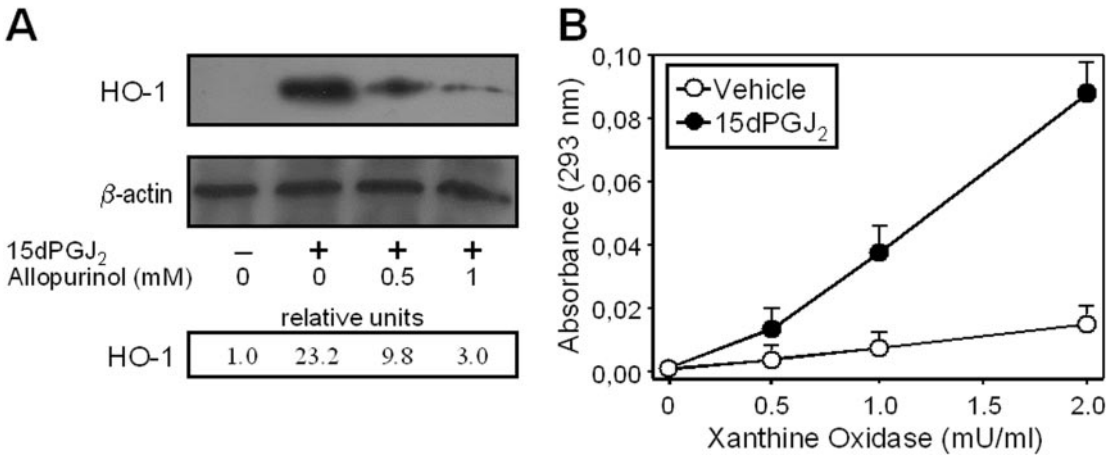

FIG. 7. **Effects of allopurinol on 15dPGJ$_2$-dependent HO-1 expression in human lymphocytes and of 15dPGJ$_2$ on xanthine oxidase activity.** *A*, cells were preincubated at 37 °C with the indicated doses of allopurinol for 1 h and then treated or not with 5 μM 15dPGJ$_2$ for 18 h. Thereafter, the cells were lysed, and HO-1 and β-actin protein levels were determined by Western blotting analysis. *B*, xanthine oxidase at the indicated doses was incubated at 37 °C with vehicle (0.1% Me_2SO) or 10 μM 15dPGJ$_2$ for 20 min. Uric acid production was then measured on the basis of the absorbance at 293 nm. Plotted values are the mean ± S.E. from three separate experiments.

stream activator of extracellular signal-regulated kinases 1 and 2 (ERK1/2). When these reagents were added to cultures prior to treatment with 15dPGJ$_2$, it was found that whereas SB203580 (at 5–10 μM) canceled 15dPGJ$_2$-promoted HO-1 protein expression, PD098059 (up to 50 μM) was without effect on this process (Fig. 8*B*). Similarly, when the effect of these compounds was tested on 15dPGJ$_2$-dependent HO-1 mRNA synthesis, both LY294002 and SB203580 abolished this process, whereas PD098059 lacked any inhibitory action (Fig. 8*C*). Finally, the effects of genistein and staurosporine (two nonspecific inhibitors of protein tyrosine kinases and protein kinase C, respectively), as well as of cyclosporin A and okadaic acid (two specific inhibitors of protein phosphatases PP-2B and PP-1/2A, respectively), were also tested. It was found that all these compounds failed to inhibit 15dPGJ$_2$-dependent HO-1 protein synthesis (data not shown). Therefore, our data indicate that activation of the PI3-kinase and p38 MAP kinase pathways are upstream signaling events required for 15dPGJ$_2$-dependent HO-1 expression.

15dPGJ$_2$ Induction of HO-1 Expression Is Dependent on NF-κB—15dPGJ$_2$ has been reported to elicit the activation of the transcription factor NF-κB in human T cells (22). Also, an NF-κB-binding motif has been found in the promoter of the mouse *Ho-1* gene (58). In order to assess whether NF-κB activation is essential for the induction of HO-1 expression by 15dPGJ$_2$ in human lymphocytes, the NF-κB-blocking peptide SN50 was used. This peptide inhibits nuclear translocation and thereby the intranuclear functions of NF-κB (59, 60). Fig. 9 clearly shows that in human lymphocytes treated with the SN50 peptide prior to the addition of 15dPGJ$_2$, induction of HO-1 protein expression was reduced by about 4-fold. As a control, the effect of the mutant SN50 peptide (SN50M), which is able to bind NF-κB but does not inhibit its nuclear translocation, was also tested. As also shown in Fig. 9, the mutant SN50M peptide had no effect on induction of HO-1 synthesis by 15dPGJ$_2$. These data indirectly suggest that 15dPGJ$_2$-dependent HO-1 expression in human lymphocytes is dependent on transcriptional activation by NF-κB.

DISCUSSION

Prostaglandin 15dPGJ$_2$ is considered to be an anti-inflammatory molecule with therapeutic properties (1, 16, 17), which regulates cytokine secretion (8, 61) and induces apoptosis in T lymphocytes (7, 35). We have experimentally tested the possibility that 15dPGJ$_2$ could exert its anti-inflammatory effects in human lymphocytes through the expression of HO-1, a potent immunomodulator molecule (27, 28). The present study documents for the first time that 15dPGJ$_2$ elicits at micromolar (2.5–10 μM) concentrations in human lymphocytes a series of events involving the induction of *HO-1* gene expression at both mRNA and protein levels. This induction is sensitive to the inhibitor of transcription, actinomycin D, and to the protein synthesis inhibitor, cycloheximide, indicating that 15dPGJ$_2$ activates *HO-1* gene expression through modulation of transcription and/or stabilization of HO-1 mRNA. We also provide experimental evidence that 15dPGJ$_2$-dependent HO-1 induction is a process independent on PPAR-γ activation and is mediated by intracellular ROS, because it becomes enhanced by Fenton chemistry-derived ROS and, in an opposite fashion, significantly prevented by ROS scavengers, such as NAC (0.5–5 mM), TEMPO (100 μM), and Me_2SO (0.5%). In this context, we have also found that allopurinol (1 mM), an inhibitor of xanthine oxidase activity, attenuates 15dPGJ$_2$-dependent HO-1 expression and that 15dPGJ$_2$ directly enhances xanthine oxidase activity in a cell-free system. Finally, activations of the PI3-kinase and p38 MAP kinase signaling pathways and activation of the transcription factor NF-κB are shown to be intracellular events mediating 15dPGJ$_2$-dependent HO-1 expression, given that specific inhibitors of these proteins efficiently prevent HO-1 synthesis.

Previous reports (62) have not established whether 15dPGJ$_2$ elicits its anti-inflammatory effects in a PPAR-γ-dependent or -independent manner. However, it has been shown recently that 15dPGJ$_2$ still exerts anti-inflammatory actions in PPAR-γ deficient mice (15). In this light, our results indicate that 15dPGJ$_2$-promoted induction of HO-1 expression in human lymphocytes involves PPAR-γ-independent mechanisms, because classical ligands of PPAR-γ, such as troglitazone and ciglitazone, fail to elicit HO-1 protein (Fig. 2*A*) or mRNA (Fig. 2*B*) synthesis. Moreover, we have observed that other cyclopentenone prostaglandins, such as PGD_2 (the precursor of 15dPGJ$_2$) and PGA_1 and PGA_2, which do not interact with PPAR-γ or any other nuclear receptor of the PPAR subfamily, are also able to induce HO-1 mRNA expression (Fig. 2*D*) and that PGD_2 and PGA_1, but not PGA_2, also enhanced HO-1 protein synthesis (Fig. 2*C*). These results confirm that mechanisms of HO-1 induction exist in human lymphocytes that are PPAR-independent, and raise the possibility that 15dPGJ$_2$ can act through mechanisms unrelated to its binding to receptors of

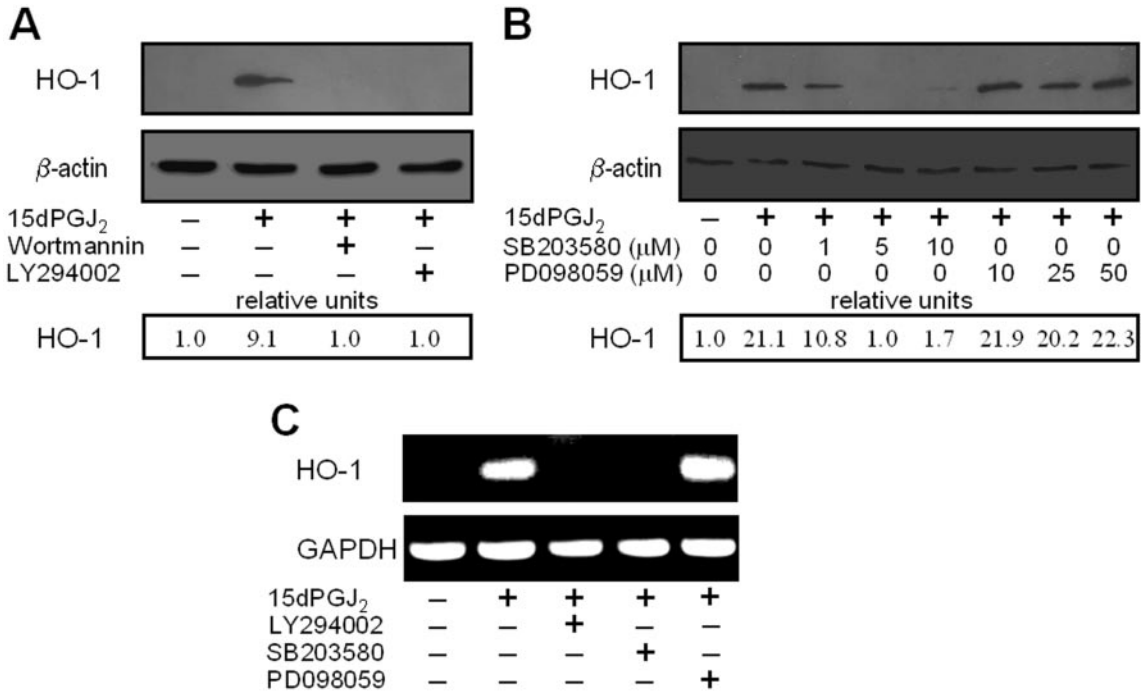

FIG. 8. **Effect of PI3-kinase and MAP kinase inhibitors on 15dPGJ$_2$-dependent HO-1 expression in human lymphocytes.** Cells were preincubated at 37 °C for 30 min in the absence or presence of 100 nM wortmannin, 20 μM LY294002, 5 μM SB203580, or 25 μM PD098059 and then treated or not with 10 μM 15dPGJ$_2$ for 4 h (*A*) or 5 μM 15dPGJ$_2$ for 4 h (*C*), or else preincubated with the indicated doses of SB203580 or PD098059 for 2 h and then treated or not with 5 μM 15dPGJ$_2$ for 18 h (*B*). Thereafter, the cells were lysed, and HO-1 and β-actin protein levels were determined by Western blotting analysis (*A* and *B*), and HO-1 and GAPDH mRNA levels were analyzed by RT-PCR (*C*).

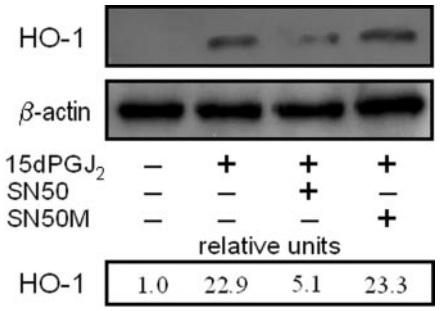

FIG. 9. **Effect of NF-κB-blocking peptide SN50 on 15dPGJ$_2$-dependent HO-1 expression in human lymphocytes.** Cells were preincubated at 37 °C in the absence or presence of 13.5 μM SN50 or SN50M peptides for 2 h, and then treated or not with 5 μM 15dPGJ$_2$ for 18 h. Thereafter, the cells were lysed, and HO-1 and β-actin protein levels were determined by Western blotting analysis.

the PPAR subfamily to elicit expression of the *HO-1* gene.

Regarding the potential implication of ROS in 15dPGJ$_2$-dependent HO-1 expression, we have found that ROS scavengers, such as NAC, TEMPO, and Me_2SO, hinder the positive effect of 15dPGJ$_2$ on HO-1 expression in human lymphocytes (Figs. 3*A* and 4). Moreover, when lymphocytes are preincubated with BSO, a glutathione synthesis inhibitor, 15dPGJ$_2$-dependent HO-1 expression levels are higher than in BSO-untreated cells (Fig. 3*B*). Furthermore, we have shown that 15dPGJ$_2$ is able to enhance intracellular ROS production in human lymphocytes, with little but still significant difference as compared with control cells (Fig. 5). Here it must be noted that when lymphocytes are incubated with vehicle alone (0.1% Me_2SO), an unspecific enhancement of ROS production is detected. Although we lack a clear explanation for this fact, it may be suggested that the contact of cells with a plastic surface during their isolation might be sufficient to elicit a partial intracellular oxidative state. This unspecific rise in ROS levels contrasts with classical observations in resting neutrophils, which in the absence of stimuli display a very low ROS production rate (63). However, in agreement with present data, a necessity for caution has been raised when using the DCFDA assay to measure intracellular ROS production by leukocytic mixed populations (54), in the sense that, given the limited intrinsic capacity of lymphocytes to synthesize ROS (64), other enzyme systems generating ROS not readily accessible to DCFDA could be operative. In this context, it has been suggested recently that ROS generated by lymphocytes include H_2O_2, singlet oxygen, and $^{\cdot}OH$, but not $O_2^{\cdot-}$ (53), thus indicating that at variance with other circulating leukocytes (*e.g.* neutrophils) lymphocytes do not exhibit potent enzyme systems for the promotion of intracellular oxidative stress (65). Regarding Fenton chemistry, we have demonstrated that the addition of Fe^{2+} and Cu^{2+} ions notably enhances 15dPGJ$_2$-dependent HO-1 expression and, in contrast, 1,10-phenanthroline, a chelator of copper and iron ions, abolishes this process (Fig. 6). Collectively, these results strongly suggest that the positive effect of 15dPGJ$_2$ on HO-1 expression requires the participation of intracellular ROS, and that the oxygen radicals seemingly involved include $O_2^{\cdot-}$ and other species generated via Fenton reactions, such as $^{\cdot}OH$. Further experiments have highlighted the possibility that cellular xanthine oxidase could also constitute a main source of the ROS acting as mediators of 15dPGJ$_2$-dependent HO-1 expression. This hypothesis is supported both by the inhibitory effect of allopurinol on this process and by the fact that 15dPGJ$_2$ promotes an increase of xanthine oxidase activity *in vitro* (Fig. 7). To our knowledge, this constitutes the first report of a direct, positive effect of 15dPGJ$_2$ on an enzyme system. Other sources of ROS, such as NADPH oxidase, cytochrome P-450, and the mitochondrial respiratory chain, which are clearly associated with the production of ROS in most cell types (53, 54), are not likely to be involved in the modulation of HO-1 production, because their respective inhibitors (HMAP, ketoconazole, and rotenone) fail to affect 15dPGJ$_2$-dependent HO-1 expression (data not shown). Taken together, our results are in

agreement with previous studies postulating HO-1 expression as an important cytoprotective mechanism against oxidative stress. In this light, a common denominator characterizing the prompt stimulation of HO-1 occurring under most circumstances, such as hypoxia, UVA radiation, and other forms of oxidative stress, is a drastic change in the intracellular redox status with a transient decrease in cellular reduced glutathione levels (25, 30). Likewise, recent studies (11, 46) have established a link between intracellular oxidative stress and the effects elicited by 15dPGJ$_2$, such as the activation of MAP kinase cascades in astrocytes and preadipocytes, or the generation of protein-bound lipid peroxidation products and the depletion of glutathione and glutathione peroxidase in human neuroblastoma cells.

The existence of a relationship between the elevation of ROS levels and the activation of downstream signaling routes, such as the PI3-kinase and MAP kinase pathways (42, 57), is also well accepted. Additionally, it has been shown recently that oxygen constitutes the upstream elements of radicals necessary for the activation of MAP kinases in response to 15dPGJ$_2$ in astrocytes and preadipocytes (11). Our results show that the p38 MAP kinase inhibitor, SB203580, is able to prevent in a dose-dependent manner 15dPGJ$_2$-dependent HO-1 expression, whereas the MEK1/2 inhibitor, PD098059, does not have such an effect (Fig. 8). This suggests that activation of the p38 MAP kinase, but not of ERK1/2, constitutes an upstream signal necessary for 15dPGJ$_2$-dependent HO-1 expression. Also, when human lymphocytes are preincubated with PI3 kinase inhibitors (wortmannin or LY294002), 15dPGJ$_2$-dependent HO-1 expression fails to take place (Fig. 8), thus highlighting the involvement of a PI3 kinase-dependent pathway in this process. Other protein kinases, such as tyrosine kinases and protein kinase C or the protein phosphatases 2A and 2B, do not appear to mediate the modulation of HO-1 expression by 15dPGJ$_2$, because their inhibitors are unable to affect 15dPGJ$_2$-dependent HO-1 synthesis (data not shown). Finally, we have shown that induction by 15dPGJ$_2$ of *HO-1* gene expression in human lymphocytes is prevented by SN50, a peptide able to block NF-κB activation, suggesting that HO-1 transcription is dependent on NF-κB (Fig. 9). This result relates, in addition to the aforementioned role of ROS in modulation of HO-1 expression, to the observation that the *HO-1* gene promoter bears a motif known as the heme-responsive element, to which NF-κB binds upon cell treatment with oxidative reagents, such as H_2O_2 (58). Furthermore, it must be emphasized that the binding of NF-κB to a number of target genes in T cells has been proven to be redox-sensitive (66). However, our observations do not allow us to discard the participation of other transcription factors as mediators of 15dPGJ$_2$ induction of *HO-1* gene expression. In this context, the factors Nrf2 and AP-1, which are also involved in HO-1 transcriptional activation (67, 68), are additional candidates to fulfill such a role.

In order to elicit its biological effects, prostaglandin 15dPGJ$_2$ requires concentrations substantially higher (up to μM) (8, 11, 17, 19, 22, 35, 46, 61) than those endogenously present at the sites of inflammation (nM levels) (2); this suggests that observations reported previously (8, 11, 17, 19, 22, 35, 46, 61) on the effects of this compound could be of pharmacological rather than physiological relevance. Collectively, our results suggest that the biological effects of 15dPGJ$_2$ at pharmacological doses on human lymphocytes involve, at least in part, the expression of a well established anti-inflammatory mediator, HO-1, which is induced by 15dPGJ$_2$ through PPAR-γ-independent mechanisms. The likely participation of ROS in this process strengthens current hypotheses establishing a link between intracellular oxidative stress and the effects elicited by 15dPGJ$_2$ on its target cells, such as the activation of MAP kinase cascades, the generation of protein-bound lipid peroxidation products, and the reduction of glutathione and glutathione peroxidase levels (11, 46).

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